Chemical Properties of Thiolated and Succinylated Caseins

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Gelation of casein may be improved by introduction of functional groups that are both hydrophilic and capable of forming disulfide bonds. Thiolated whole caseins were prepared with *S*-acetylmercaptosuccinic anhydride at four concentrations to study chemical properties related to gelation. Succinylated samples were prepared for comparison. Degree of modification was determined by decrease in reactive lysine content. Urea–PAGE showed decreased positive charge for modified proteins as degree of modification increased. In addition, α_{S1}- and β-casein bands remained while κ- and α_{S2}-casein bands disappeared compared to whole native casein. SDS–PAGE without added mercaptoethanol indicated formation of intermolecular disulfide bonds in the thiolated derivatives. All modified caseins precipitated in the presence of Ca^{2+}, but the concentration needed increased with degree of modification. Thiolated caseins were less soluble in the presence of high Ca^{2+} levels than succinylated or native caseins. Changes observed indicate successful introduction of groups that may enhance gelation.

INTRODUCTION

Caseins serve as functional as well as nutritive food additives, contributing adhesive, emulsion, coagulation, or viscoelastic properties to foods by means of hydrophobic associations and electrostatic interactions (Kinsella, 1982). However, caseins lack the ability to form extended protein structures stabilized by disulfide bonds because κ-casein and α_{S2}-casein, which comprise about 25% of the caseins, contain only two cysteine residues each and α_{S1}-casein and β-casein, which comprise about 75% of the caseins, contain none (Eigel et al., 1984). Sulfhydryl groups can be added to proteins by thiolating reagents (Kester and Richardson, 1984), and acidic groups and thiol groups can be added by *S*-acetylmercaptosuccinic anhydride (Klotz and Heiney, 1962). Gelation (Cheftel et al., 1985), dough development (Kinsella, 1982), and foam stability (Okumura et al., 1989, 1990) have all been related to disulfide bond formation.

Thiolated casein derivatives have been prepared. Singh and Fox (1989) demonstrated improved micelle heat stability when milk was treated with 2-iminothiolane, and Okumura et al. (1989, 1990) prepared thiolated α_{S1}-casein to illustrate the role of disulfide bond formation in foam stability. Bovine serum albumin was thiolated with *N*-acetylhomocysteine thiolactone, but the modified protein showed impaired whipping and gelling properties (Murphy and Howell, 1990). β-Lactoglobulin thiolated with *S*-acetylmercaptosuccinic anhydride at 14–16 sulfhydryl (SH) groups/dimer formed soluble polymers upon oxidation and gels upon treatment with Ca^{2+}; when thiolated with either *N*-acetylhomocysteine thiolactone or *S*-acetylmercaptosuccinic anhydride, β-lactoglobulin formed gels upon oxidation at 18 SH groups/dimer (Kim et al., 1990).

The purpose of our experiments was to compare the chemical properties of thiolated caseins and succinylated caseins with the goal of enhancing casein functionality as a food ingredient.

MATERIALS AND METHODS

Whole native caseins were isolated from freshly skimmed mixed herd milk by isoelectric precipitation with 1 N HCl at pH 4.6 and 30 °C. The acid curd was separated from the whey by filtration through a double layer of cheesecloth, washed with 1 volume of acidified water, and redispersed in a volume of water equal to the original volume of milk using 1 N NaOH to adjust the pH of the casein mixture to 7. The dispersed casein was then filtered through Whatman No. 541 paper to remove any insoluble particles. This procedure was repeated once, and the casein was subsequently freeze-dried.

Casein Derivatives. Four thiolated casein derivatives were prepared (Klotz and Heiney, 1959, 1962) by reacting *S*-acetylmercaptosuccinic anhydride (SAMSA) with whole native casein in molar ratios of 0.5:1, 1:1, 2:1, and 4:1 SAMSA:total casein lysine residues. The average molecular weight of whole native casein was calculated to be 23 300 containing 13.5 residues of lysine by assuming a ratio of 1:3:3:1 κ:β:α_{S1}:α_{S2} caseins (Eigel et al., 1984).

Freeze-dried whole native casein (2.5 wt %) was prepared in 0.2 M phosphate buffer, pH 7.0. SAMSA was added as a solid to the constantly stirring casein solution. The pH was continuously adjusted to 7.0 by the addition of 1 N NaOH. The reaction was allowed to proceed for 1 h at room temperature and the solution filtered through Whatman No. 541 paper. In preliminary experiments, the thiolated caseins were deacetylated by adding NH_2OH·HCl (hydroxylamine hydrochloride) in 10-fold excess of lysine residues and adjusting the pH of the reaction mixture to 7 by addition of 1 N NaOH. After 10 min, the reaction mixture was desalted by passage through a Sephadex G-25 column using water as the eluant and freeze-dried.

Thiolated derivatives used for most experiments were not deacetylated. Immediately after reaction with SAMSA and filtration through Whatman No. 541 paper, the pH of the filtrate was decreased to 3 with 1 N HCl to precipitate the modified casein and to stop the reaction. The precipitate was separated from the supernate by centrifugation for 10 min at 1000*g* and redissolved by suspension in 400 mL of water and addition of 1 N NaOH to pH 7.0. After filtration through Whatman No. 541 paper, the modified casein solution was freeze-dried. Succinylated casein derivatives were prepared in a similar manner.

Analysis. Reactive lysine was measured by the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method of Kakade and Liener (1969) as modified by Posati et al. (1972). ϵ-TNP-L-Lys HCl·H_2O was used to construct standard curves for both 346 nm, as recommended by Kakade and Liener (1969), and 420 nm, another ϵ-TNP-L-Lys maximum. There were advantages to both wavelengths. At 346 nm, the extinction coefficient (1.82A/μmol) for TNP-Lys was higher than that at 420 nm (0.8A/μmol), but there was more interference from the reagent blank. The reactive lysine values were calculated from absorbances at 420 nm. Protein content of the casein samples was determined by absorbance at 280 nm.

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Sulfhydryl and disulfide contents of casein, thiolated caseins, and succinylated caseins were measured according to the method of Beveridge et al. (1974). Modifications were made for determination of the disulfide content as follows: the TRIS–glycine urea buffer, used to dissolve the reduced protein after TCA precipitation and washing to remove excess mercaptoethanol, was pH 8.8 rather than 8.0, and the protein content of each disulfide sample was measured by absorbance at 280 nm or BCA (Pierce Chemical Co.) assay, just before the addition of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). An extinction coefficient of 13 600 was used to calculate sulfydryl content.

Solubility. Aliquots of 1 M $CaCl_2$ in water were added to 0.2% (w/w) solutions of the samples in 0.01 M imidazole, pH 7. Sample solutions were prepared and allowed to hydrolyze in the imidazole for 12 h to remove the acetyl group from the thiol. After addition of Ca^{2+}, centrifugation at 100000*g* at 37 °C for 0.5 h was necessary to remove the very fine calcium caseinate aggregates formed. Protein concentration in the supernate was measured by difference in absorbance at 280 nm from a baseline generated by extrapolation of absorbance values between 340 and 250 nm. Curves of absorbance vs concentration were generated by serial dilutions of the original casein–imidazole solution.

The solubility of whole and modified caseins at various pHs was determined by preparing 0.2% (w/w) casein solutions in deionized water. Each solution was adjusted to the desired pH by the addition of 0.1 N HCl. At each pH, samples were removed for protein determination at 280 nm after centrifugation at 8800*g* for 15 min.

Electrophoresis. SDS- and urea–PAGE electrophoretic patterns of whole native casein and modified caseins were obtained with the PhastSystem as described by Strange et al. (1991) with the following exceptions. For SDS–PAGE, the sample solvent used was 60 mM Tris-HCl, 0.5 mM EDTA, 3.5% SDS, pH 8, with 10% 2-mercaptoethanol as needed. The electrophoresis was run for 70 Vh. Concentrations of the molecular weight standards (Sigma Chemical Co., St. Louis, MO), whole native casein, and modified caseins were 2, 1, and 1.5 μg/μL, respectively. For urea–PAGE, sample concentration was 0.5 μg, 2-mercaptoethanol was 10%, and the electrophoretic conditions were 15 °C, 300 V, 7.5 mA, and 2.5 W for a total of 110 Vh.

HPLC. Reversed-phase HPLC separations of whole and modified caseins were determined on a 22-cm C_8 column as described by Strange et al. (1991). Assignments to α_{S1}- or β-casein were made on the basis of retention time and the relationships of the peaks to each other. α_{S1}-Casein eluted before β-casein.

RESULTS AND DISCUSSION

Chemical modification of food proteins to improve their nutritional and functional properties has been an area of expanding research interest. The most common chemical modification used for food proteins has been the acylation of amino acid residues, particularly lysine. Succinylation introduces anionic succinate residues covalently linked to the ε-amino group of lysine. Electrostatic repulsive forces resulting from the enhanced negative charge may lead to more extensive unfolding of the polypeptide chain. Altered functionality brought about by succinylation includes increased aqueous solubility, altered viscosity, and modified surfactant properties such as emulsification and foaming (Nakai and Li-Chan, 1989). Since the caseins have few cysteine residues, the introduction of additional sulfhydryl groups by acylation with SAMSA, along with the addition of negatively charged carboxyl groups, suggested a way to modify casein (Figure 1). Enhanced functional attributes associated with sulfhydryl content include gelation, viscosity, elasticity, and texture formation during fiber spinning, as well as retention of benefits from the negative charge contributed by the addition of free carboxyl groups (Kester and Richardson, 1984).

Table I shows that the amount of modification increased with the reagent:lysine ratio. Klotz and Heiney (1962) reported 23 and 61% thiolation for SAMSA:lysine ratios

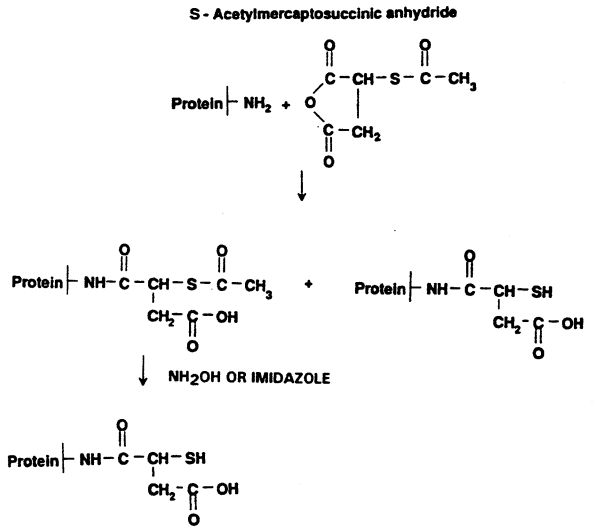

Figure 1. Reaction of *S*-acetylmercaptosuccinic anhydride with the ε-amino group of lysine.

Table I. Modification of Lysine by SAMSA or Succinic Anhydride

mole ratio[a]	% thiolation	% succinylation
0.5:1	19	6
1:1	34	12
2:1	54	40
4:1	65	75

[a] Reagent:lysine ratio in moles per mole.

of 0.5:1 and 4:1, respectively, for bovine serum albumin. Kim et al. (1990) found 70 and 80% modification at SAMSA:lysine ratios of 2:1 and 5:1, respectively, for β-lactoglobulin. We thiolated whole casein to levels comparable to those for bovine serum albumin but less than those for β-lactoglobulin.

Hoagland (1966, 1968) reported 86% succinylation for β-casein at 1:1 and 0.2:1 weight ratios of succinic anhydride to casein. We succinylated whole casein with weight ratios comparable to those used by Hoagland (1968) for β-casein and got comparable levels of succinylation. SAMSA reacted with lysine more readily than succinic anhydride at every ratio level except 4:1 reagent:lysine; at that level succinic anhydride reacted with 75% of lysine residues, while SAMSA reacted with 65% of lysine residues. However, these values were obtained for only one series of reactions, and the actual differences were small. Others (Groninger, 1973; Muhlard et al., 1968) have noted that large excesses of succinic anhydride were necessary to obtain high levels of succinylation.

Table II shows that, for fully deacetylated thiolated caseins (prepared in preliminary experiments), free sulfhydryl content was low and disulfide bonds were present. However, the number of sulfhydryl and disulfide groups measured did not increase as the ratio of reagent to lysine increased, indicating either that disulfide bonds are not fully reduced by mercaptoethanol or that disulfide bonds re-formed during the manipulations to remove the mercaptoethanol in the assay. Analysis of the partly acetylated thiolated caseins showed much higher free sulfhydryl levels, but when attempts were made to measure disulfide bonds, a decrease in the SH content was noted. This indicated that added sulfhydryl groups in thiolated casein are very readily oxidized. Natural disulfide bonds, i.e., those present in the α_{S2}- and κ-caseins in whole native casein and succinylated casein, do not appear to be as labile. The sulfhydryl and disulfide content (as sulfhydryl) of whole native casein and succinylated caseins is about

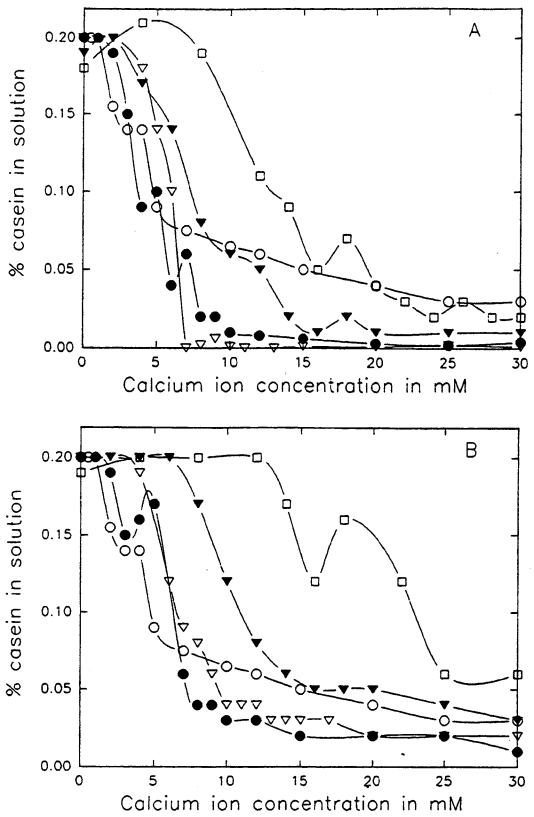

Figure 2. Effect of thiolation and succinylation on the Ca^{2+} solubility of casein: (○) whole native casein; (●) 0.5:1 reagent:lysine ratio in moles per mole; (▽) 1:1 reagent:lysine ratio in moles per mole; (▼) 2:1 reagent:lysine ratio in moles per mole; (□) 4:1 reagent:lysine ratio in moles per mole. (A) Thiolated caseins—reagent is *S*-acetylmercaptosuccinic anhydride. (B) Succinylated caseins—reagent is succinic anhydride.

Table II. Sulfhydryl and Disulfide Groups of Caseins and Casein Derivatives

protein	sulfhydryl/ mol of casein	sulfhydryl and disulfide (as sulfhydryl)/ mol of casein
whole native casein	0.07 ± 0.03 (N = 12)	0.35 ± 0.05 (N = 12)
deacetylated thiolated casein		
SAMSA:casein		
0.5:1	0.51 ± 0.02 (N = 3)	1.56 ± 0.07 (N = 3)
1:1	0.57 ± 0.02 (N = 3)	5.37 ± 1.03 (N = 6)
2:1	0.61 ± 0.03 (N = 4)	4.16 ± 0.58 (N = 8)
4:1	0.10 ± 0 (N = 3)	2.73 ± 0.33 (N = 3)
thiolated casein		
SAMSA:casein		
0.5:1	1.40 ± 0.09 (N = 6)	0.79 ± 0.21 (N = 6)
1:1	1.98 ± 0.10 (N = 6)	1.12 ± 0.12 (N = 6)
2:1	4.32 ± 0.36 (N = 6)	1.62 ± 0.25 (N = 6)
4:1	6.02 ± 0.20 (N = 6)	2.61 ± 0.98 (N = 6)
succinylated casein		
succinic anhydride:casein		
0.5:1	0.03 ± 0.01 (N = 3)	0.30 ± 0.02 (N = 3)
1:1	0.02 ± 0.02 (N = 3)	0.31 ± 0.02 (N = 3)
2:1	0.04 ± 0.01 (N = 3)	0.28 ± 0.01 (N = 3)
4:1	0.03 ± 0.01 (N = 3)	0.28 ± 0.03 (N = 3)

0.5 mol of SH/mol of casein, as calculated from information presented in Eigel et al. (1984). We measured 0.35 and 0.29 mol of SH/mol of casein, respectively.

Since it is the formation of intermolecular cross-links that is the driving force for gel formation, caseins that have a large content of free sulfhydryl, rather than disulfide bonds, would have the greatest potential for gelation.

Solubility. Solubilities in the presence of Ca^{2+} of both thiolated and succinylated caseins are shown in parts A and B, respectively, of Figure 2, compared to that of untreated whole native casein. The thiolated caseins began to precipitate at higher Ca^{2+} concentrations than whole native casein. The casein solubility decreased 25%, i.e., 0.15% casein remains in solution, when the Ca^{2+} concentration reached 2 mM for whole native casein, 3 mM for 0.5:1, 5 mM for 1:1, 6 mM for 2:1, and 10 mM for 4:1 thiolated caseins (Figure 2A). Whole native casein showed a plateau in the Ca^{2+} solubility curve between 2 and 4 mM Ca^{2+} because β-casein is less sensitive than α_{S1}-casein to Ca^{2+} precipitation. Such a plateau was also evident between 5 and 6 mM Ca^{2+} for 0.5:1 thiolated casein but not for 1:1 tholated casein. The Ca^{2+} solubility curves for the other thiolated caseins are so distorted from that of whole native casein that interpretation of any of the minor fluctuations in the smoothness of the curves is precluded. At higher Ca^{2+} concentrations, thiolated caseins were less soluble than whole native casein. At 20 mM Ca^{2+}, only 4:1 thiolated casein had a solubility equivalent to that of whole native casein.

The succinylated caseins also began to precipitate at higher Ca^{2+} levels than did whole native casein (Figure 2B). The casein solubility decreased 25% when Ca^{2+} concentration reached 3 mM for 0.5:1, 5 mM for 1:1, 9 mM for 2:1, and 15 mM for 4:1 succinylated caseins. A plateau in the Ca^{2+} solubility curve for 0.5:1 succinylated casein between 3 and 5 mM Ca^{2+} was also evident. At higher Ca^{2+} concentrations, 0.5:1 and 1:1 succinylated caseins were less soluble than whole native casein; 2:1 succinylated casein and whole native casein had equivalent solubilities; and 4:1 succinylated casein was much more soluble.

Comparison of Ca^{2+} solubility of thiolated and succinylated caseins showed several differences. Thiolated caseins with low levels of substitution [0.5:1 and 1:1 mole ratios with 19 and 34% substitution, respectively (Table I)] began precipitating at Ca^{2+} levels comparable to those of 0.5:1 and 1:1 succinylated caseins (6 and 12% substitution, respectively). At higher levels of substitution, thiolated caseins with 54 (2:1) and 65% (4:1) substitution required less Ca^{2+} to initiate precipitation (6 and 10 mM) than did succinylated caseins with 40 (2:1) and 75% (4:1) substitution (9 and 15 mM).

The additional acid groups in both thiolated and succinylated caseins cause an increase in the amount of Ca^{2+} needed to initiate precipitation compared to whole native casein. At Ca^{2+} levels greater than those needed to initiate precipitation, thiolated caseins were less soluble than whole native casein, while succinylated caseins were more soluble or less soluble depending on the percentage of lysines substituted. These results confirm those of Girerd et al. (1984) and Hoagland (1966), who noted increases in the amount of Ca^{2+} needed to precipitate succinylated caseins and attributed this increase to the binding of Ca^{2+} to the additional negatively charged acid groups in the succinylated casein. Hoagland (1966) showed that highly succinylated β-casein did not precipitate with high levels of Ca^{2+} and attributed this observation to the effectiveness of the high net negative charge of the modified caseins in preventing aggregation and subsequent precipitation. The less modified succinylated caseins behaved differently than did whole native casein or the 4:1 succinylated casein when treated with Ca^{2+}. Upon centrifugation, the pellets were hard and clear and the supernates exhibited very little light scatter, in sharp contrast to the native casein and the 4:1 succinylated caseins. The opalescence of the native casein was due to incomplete aggregation and to the resistance of α_S-casein and β-casein in the presence of κ-casein to precipitation by Ca^{2+} ion (Dalgliesh, 1982). The opalescence of the 4:1 succinylated casein, which occurred at higher Ca^{2+} levels,

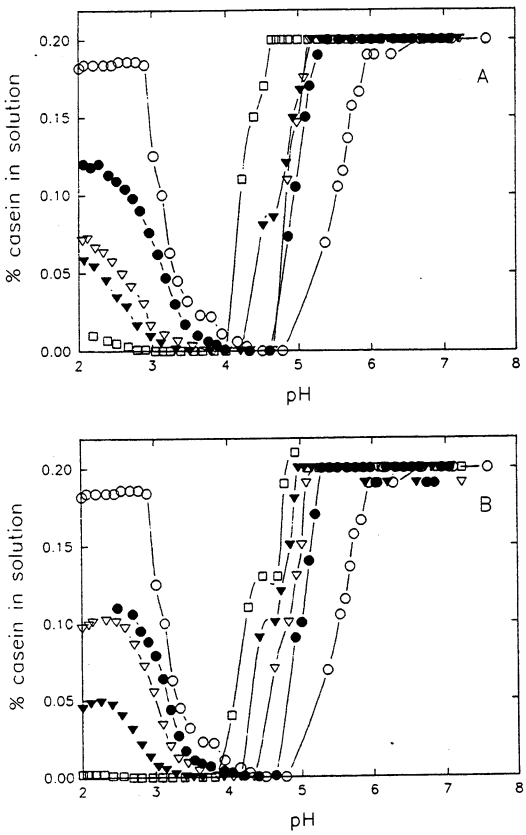

Figure 3. Effect of pH on solubility of thiolated (A) and succinylated (B) caseins: (○) whole native casein; (●) 0.5:1 reagent:lysine ratio in moles per mole; (▽) 1:1 reagent:lysine ratio in moles per mole; (▼) 2:1 reagent:lysine ratio in moles per mole; (□) 4:1 reagent:lysine ratio in moles per mole. (A) Thiolated caseins—reagent is *S*-acetylmercaptosuccinic anhydride. (B) Succinylated caseins—reagent is succinic anhydride.

may have been due to slower aggregation of the casein molecules because of the high net negative charge resulting from modification.

The effects of pH on solubility for the two sets of modified caseins are shown in Figure 3. In both cases, as the degree of modification increased, the pH at which the casein precipitated decreased, as did the amount of casein resolubilized at low pH (≤3). No differences were noted between partly deacetylated and fully deacetylated reduced thiolated caseins. The minor irregularities in the curves are due to experimental variations in protein assays on highly opalescent samples. The amount of casein that resolubilized at acid pH was extremely sensitive to degree of modification. For example, 19% thiolation (0.5:1) and 6% succinylation (0.5:1) decreased solubility by 40% at pH 2.4 while causing less than 10% change in the pH at which precipitation occurred.

An important desirable functional effect of succinylation is increased solubility of proteins (Chen et al., 1975; Paulson and Tung, 1987; Franzen and Kinsella, 1976a; Beuchat, 1977). Other researchers have reported no major changes in initial pH of precipitation at low levels of modification (Girerd, 1984; Sato and Nakamura, 1977). Our results showed slight reductions in the pH at which less modified thiolated and succinylated caseins precipitated and greater changes at high levels of modification.

The solubility of proteins at acid pH is dependent on the number of basic amino acid residues present (Cheftel et al., 1985); if lysine residues are modified, solubility will decrease at low pH. This has been demonstrated for succinylated proteins (Beuchat, 1977; Sato and Nakamura, 1977; Chen et al., 1975; Franzen and Kinsella, 1976a). Our succinylated and thiolated caseins behaved in a similar manner. Others (Girerd et al., 1984; Franzen and Kinsella, 1976b) showed decreased solubility at low pH only after extensive succinylation.

SDS–PAGE. The SDS–PAGE gel patterns of the thiolated, succinylated, and whole caseins are shown in Figure 4. These samples were run both without and with the addition of 2-mercaptoethanol as a disulfide-reducing agent. The patterns of the modified caseins showed that no significant degradation of the caseins occurred during the modification processes. The SDS patterns of the 0.5:1 and 1:1 succinylated caseins (Figure 4A, lanes 4–7) showed that although there were two major fractions present, as in whole native casein (Figure 4A, lanes 2 and 3), the definition of the bands decreased as the amount of modification increased and there was no significant increase in molecular weight of these modified caseins. Treatment with 2-mercaptoethanol had little effect on the gel patterns (Figure 4A, lanes 3, 5, and 7).

The patterns of the 2:1 and 4:1 succinylated caseins (Figure 4B, lanes 4–7) showed greater changes from whole native casein (Figure 4B, lanes 2 and 3) than less modified casein in Figure 4A. There was only one major casein band present in unreduced succinylated caseins (Figure 4B, lanes 4 and 6); however, in the reduced succinylated caseins (Figure 4B, lanes 5 and 7), a smaller band with molecular weight similar to that of β-casein in whole native casein (Figure 4B, lanes 2 and 3) appeared. The apparent molecular weight of the highly modified succinylated casein increased slightly and the staining with Coomassie blue decreased as the degree of modification increased (Figure 4B, lanes 6 and 7) due to loss of positive charges for the dye to bind.

The patterns of the 0.5:1 and 1:1 thiolated caseins (Figure 4C, lanes 4–7) showed the two major casein bands as in whole native casein. These bands did not lose as much definition with increasing modification as the succinylated caseins. The unreduced SDS patterns of the thiolated caseins (lanes 4 and 6) showed very light streaking in the higher molecular weight regions of the gels, rather than the single light band evident in unreduced whole native casein and the unreduced 0.5:1 and 1:1 succinylated casein. This streaking was most evident in the 1:1 thiolated casein band (lane 6).

The patterns of the 2:1 and 4:1 thiolated caseins (Figure 4D, lanes 4–7) showed changes similar to those of the 2:1 and 4:1 succinylated caseins (Figure 4B) except that the increase in molecular weight of the major band for the thiolated casein was slightly less than the increase in molecular weight for the succinylated casein. Our thiolated caseins apparently did not form extensive intermolecular disulfide bonds even though protected (with an acetyl group) and unprotected (acetyl group hydrolyzed during synthesis reaction) sulfhydryl groups are present in these caseins (Klotz and Heiney, 1962) (Figure 1). Kim et al. (1990) showed reduced migration in SDS–PAGE gels for SAMSA-treated β-lactoglobulin but no change in migration for the same protein treated with *N*-acetylhomocysteine thiolactone; Murphy and Howell (1990) showed broadening of the major protein band in SDS–PAGE patterns of *N*-acetylhomocysteine thiolactone-treated bovine serum albumin, and Messinger et al. (1987) reported decreased mobility (apparent molecular weight increase) of succinylated corn germ protein isolate when compared with the native proteins in SDS–PAGE. Decreased migration in the SDS gels of the major bands of our thiolated and

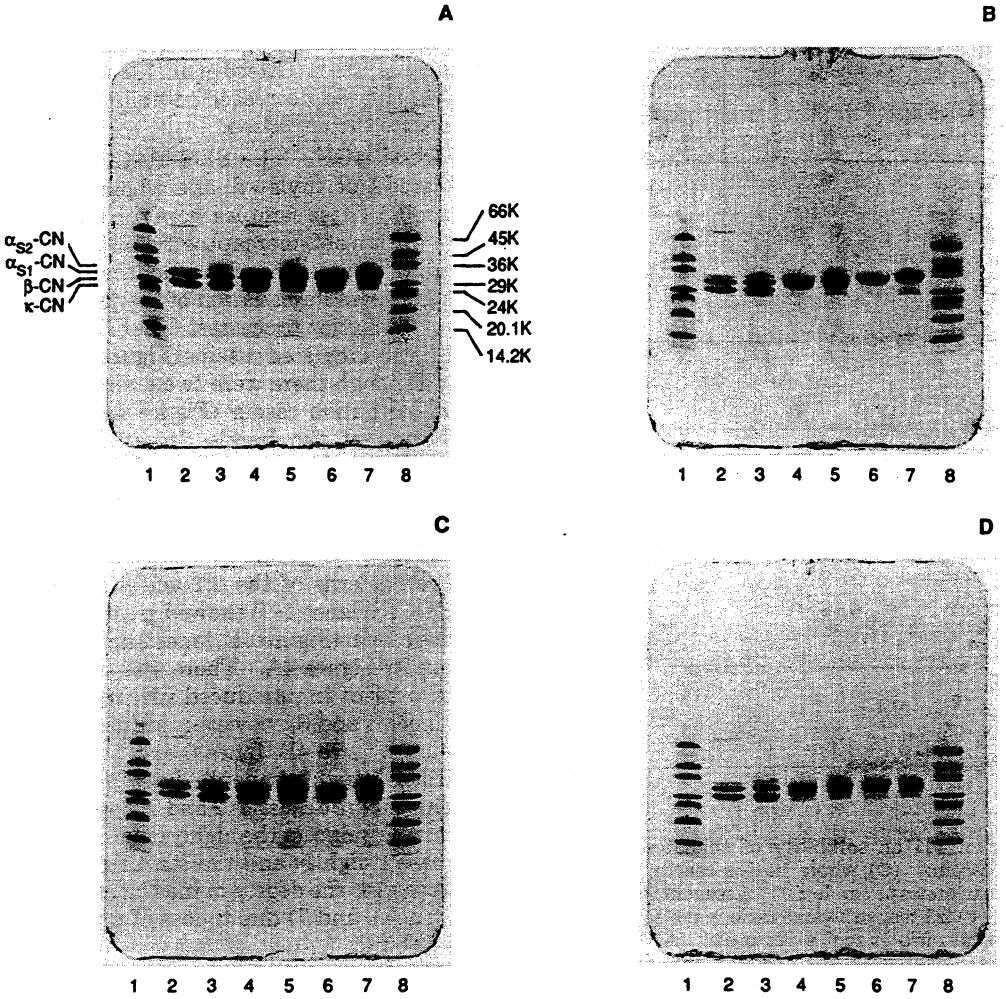

Figure 4. SDS gel electrophoresis of succinylated (A and B) and thiolated (C and D) caseins, with and without 2-mercaptoethanol. (A and C) (Lanes 1 and 8) Molecular weight standards; (lanes 2 and 3) whole native casein; (lanes 4 and 5) 0.5:1 reagent:lysine; (lanes 6 and 7) 1:1 reagent:lysine (lanes 2, 4, and 6) unreduced; (lanes 3, 5, and 7) 2-mercaptoethanol treated. (B and D) (Lanes 1 and 8) Molecular weight standards; (lanes 2 and 3) whole native casein; (lanes 4 and 5) 2:1 reagent:lysine; (lanes 6 and 7) 4:1 reagent:lysine; (lanes 2, 4, and 6) unreduced; (lanes 3, 5, and 7) 2-mercaptoethanol treated. CN, casein.

succinylated caseins was caused by the addition of carboxyl groups and concomitant swelling of the protein.

Urea–PAGE. The urea–PAGE native electrophoresis patterns for both succinylated and thiolated caseins are shown in Figure 5. The α_{S2}-casein bands disappeared from all of the modified casein patterns (lanes 3–6). The thiolated caseins (Figure 5C,D, lanes 3–6) are similar to the patterns of the succinylated caseins. Murphy and Howell (1990) showed increased anode migration of *N*-acetylhomocysteine thiolactone-treated bovine serum albumin under native PAGE conditions, but this increase was probably due to lack of lysine residues rather than an increase in acidic groups. The gel pattern of the 1:1 thiolated casein without 2-mercaptoethanol (Figure 5C, lane 5) showed more streaking and blurring, compared to the same sample treated with 2-mercaptoethanol (Figure 5C, lane 6), indicating that disulfide bonds formed in the 1:1 thiolated casein.

The succinylated caseins (Figure 5A,B, lanes 3–6) retained identifiable β- and α_{S1}-casein bands that showed greater migration toward the anode than native β- and α_{S1}-caseins (Figure 5A,B, lanes 1, 2, 7, and 8); the β-casein bands showed the greatest change. The β-casein bands in the succinylated derivatives 0.5:1 (Figure 5A, lanes 3 and 4), 1:1 (Figure 5A, lanes 5 and 6), and 2:1 (Figure 5B, lanes 3 and 4) were more diffuse than those of native β-casein and native and succinylated α_{S1}-casein. Small, separate bands within the β-casein band of the 0.5:1 and 1:1 succinylated caseins indicate that differences in charge caused by a different number of lysine residues reacting with succinic anhydride are detectable on the gel and that casein molecules with different numbers of lysines reacted are present simultaneously. As expected, succinylated caseins also displayed decreased Coomassie blue staining with increased modification. There were no major differences in the patterns of the succinylated caseins due to the presence of 2-mercaptoethanol. Hoagland (1966) demonstrated increased migration toward the anode for succinylated β-casein in urea gels, as did Sato and Nakamura (1977) for succinylated egg white protein under nondenaturing conditions.

HPLC. Reversed-phase C_8 HPLC of the succinylated caseins showed decreasing resolution (chromatograms not shown) as the degree of modification increased; retention times for the α- and β-caseins also increased (Table III). Treatment with 2-mercaptoethanol had no effect on retention times. Behavior of thiolated caseins was similar to that of succinylated caseins except that 2-mercaptoethanol decreased the retention times of both the α- and β-caseins.

Increased retention time on a reversed-phase column usually indicates an increase in hydrophobic character of the protein. The HPLC elution solvents used contained 0.1% trifluoroacetic acid and the pH was 3.2, below the

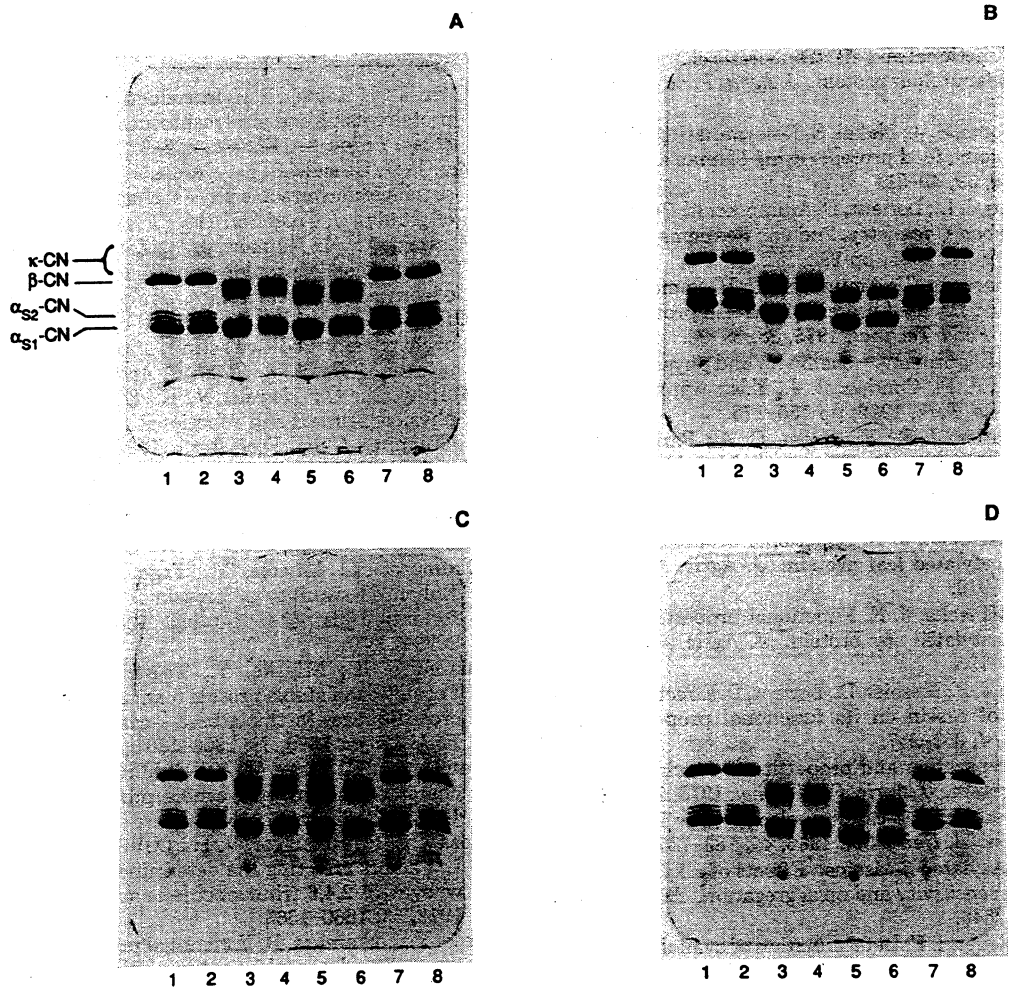

Figure 5. Urea–PAGE gel patterns of succinylated (A and B) and thiolated (C and D) caseins, with and without 2-mercaptoethanol. (A and C) (lanes 1, 2, 7, and 8) Whole native casein; (lanes 3 and 4) 0.5:1 reagent:lysine; (lanes 5 and 6) 1:1 reagent:lysine; (lanes 1, 3, 5, and 7) unreduced; (lanes 2, 4, 6, and 8) 2-mercaptoethanol treated. (B and D) (Lanes 1, 2, 7, and 8) Whole native casein; (lanes 3 and 4) 2:1 reagent:lysine; (lanes 5 and 6) 4:1 reagent:lysine; (lanes 1, 3, 5, and 7) unreduced; (lanes 2, 4, 6, and 8) 2-mercaptoethanol treated. CN, casein.

Table III. HPLC of Whole Casein, Thiolated Casein, and Succinylated Casein on a Reversed-Phase C_8 Column

	retention time, min			
	thiolated caseins		succinylated caseins	
sample	α	β	α	β
WNC[a]	17	21.5	16.9	21.6
WNC + SH[b]	17	21.4	17	21.7
0.5:1[c]	17.4	21.4	17.2	21.2
0.5:1 + SH	17.1	21.4	17.3	21.3
1:1	18.5	22.6	17.8	22
1:1 + SH	17.7	21.8	18.0	22.1
2:1	19	23.7	19	23.4
2:1 + SH	18.5	23	19.1	23.3
4:1	21.5	25.4	21	25
4:1 + SH	20.7	24.4	21	25.7

[a] Whole native casein. [b] Treated with mercaptoethanol. [c] Reagent: lysine ratio (moles per mole) used in preparation of the sample.

pK_a of amino acid carboxyl groups. None of the added carboxyl groups in the modified caseins would be charged under our HPLC conditions, possibly leading to increased hydrophobic character through conformation changes. Kim and Kinsella (1987) and Ma et al. (1986) reported increased hydrophobic character in succinylated glycinin and succinylated gluten, respectively. This increased hydrophobic character was attributed to unfolding of the native protein because of charge repulsion and exposure of interior hydrophobic residues. However, Paulson and Tung (1987) and Murphy and Howell (1990) reported decreased hydrophobic character in succinylated canola protein isolate and *N*-acetylhomocysteine thiolactone-treated bovine serum albumin, respectively.

Conclusions. One of the difficulties in evaluating the use of SAMSA as a thiolating agent was distinguishing the changes in protein chemistry related to addition of carboxyl groups and those related to creation of sulfhydryl groups. Both succinic anhydride and SAMSA are effective reagents, but SAMSA reacted more readily than did succinic anhydride at low reagent:lysine ratios, making it a more effective modification agent. Solubility in the presence of Ca^{2+} was greatly altered by both succinylation and thiolation but in different ways. Thiolated caseins were effectively aggregated at high Ca^{2+} levels and precipitated to a greater extent than native casein, while the succinylated caseins showed inhibited aggregation at high Ca^{2+} levels. Both succinylated and thiolated caseins retained sufficient characteristics of the individual proteins to be separable on urea–PAGE and on reversed-phase HPLC. Thiolated and succinylated caseins both seemed to have increased hydrophobic character, although this should be addressed by a more direct method.

Further studies are underway to evaluate the functional properties of succinylated and thiolated caseins with special emphasis on viscoelastic properties induced by disulfide formation.

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Received for review August 7, 1992. Accepted September 29, 1992. Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Registry No. Ca^{2+}, 7440-70-2.